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Chitin synthase in encysting *Entamoeba invadens*

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Although the cyst wall of *Entamoeba invadens* contains chitin, synthesis of this structural polymer during encystation has not been described before. Here we report that conditions which stimulate encystation of the parasite lead to increased chitin synthase (ChS) activity, measured by incorporation of [^{3}H]GlcNAc ([^{3}H]*N*-acetylglucosamine) from UDP-GlcNAc. The radiolabelled product was precipitable by trichloroacetic acid or ethanol and identified as chitin because it was digested by purified chitinase to radioactive chitobiose and GlcNAc. Cell fractionation indicated that approx. 60% of the enzyme is in the high-speed supernatant. pH–activity profiles showed that soluble ChS has an optimum at 6.0, whereas particulate ChS has a peak at pH 7.0–7.5. Both the activities were dependent on bivalent metal ions, especially Mn^{2+} and Mn^{2+} plus Co^{2+}. In contrast with the ChS of other organisms, neither the particulate nor the soluble ChS of *E. invadens* was activated by trypsin treatment. Soluble and particulate ChS were also stimulated by digitonin and phosphatidylserine, whereas phosphatidylethanolamine stimulated only the soluble ChS. The enzyme activities were inhibited by UDP, UDP-glucose and UDP-GalNAc, but not by the analogues Polyoxin-D or Nikkomycin. This is the first report of an enzyme which is developmentally regulated during encystation of the primitive eukaryotic genus *Entamoeba*.

INTRODUCTION

Entamoeba invadens, a protozoan parasite of reptiles, has two life-cycle stages, an infective cyst form and an amoeboid trophozoite. Cysts, unlike trophozoites, can survive outside the host for prolonged periods, since their thick resistant walls are believed to protect them from unfavourable environmental conditions and hypo-osmotic lysis. Cultured *E. invadens* trophozoites readily differentiate into cysts *in vitro* in response to glucose deprivation [1] or hypo-osmotic conditions [2]. In contrast, the related species, *E. histolytica*, the cause of amoebic dysentery and liver abscess in humans, does not routinely encyst in pure culture. Chitin, a linear polymer of $\beta 1 \rightarrow 4$-linked *N*-acetylglucosamine (GlcNAc), had previously been shown to be a major component of the cyst walls of both *E. invadens* and *E. histolytica* by X-ray diffraction [3] and lectin-binding studies [4]. In contrast, chitin was not detected in the amoebal form. Therefore study of the synthesis of chitin by *E. invadens* may also give insights into differentiation of the human parasite.

Chitin synthase I (ChS I) from *Saccharomyces cerevisiae*, which catalyses stepwise polymerization of GlcNAc from UDP-GlcNAc, has been characterized in great detail [5]. The synthesis of chitin by intact *S. cerevisiae* may be complex, because a second enzyme (ChS II) was detected [6–8] after disruption of the ChS I gene. Moreover, the recent observation [9] of chitin in cells with disruptions in both ChS I and ChS II genes points to the existence of a third ChS. ChS II has both biochemical similarities to, and differences from, ChS I, and the genes share 36% identity at the DNA level and 42% at the protein level [10]. ChS activity has also been detected in other fungi [11], worms [12] and crustacea [13], but these enzymes have not been extensively characterized. In the present study we characterize the ChS activity of *E. invadens* and show that it is developmentally regulated during encystation.

EXPERIMENTAL

Materials

Unless otherwise specified, all chemicals were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and were of the highest purity available. UDP-[^{3}H]GlcNAc (18.5 Ci·mmol^{-1}) was obtained from New England Nuclear Corp., Boston, MA, U.S.A. Polyoxin D was purchased from Calbiochem, La Jolla, CA, U.S.A., and Nikkomycin was a gift from Bayer. Cytohelicase, which hydrolyses chitobiose to GlcNAc, was generously given by Dr. Fred Hefron, Scripps Clinic, La Jolla, CA, U.S.A. [^{3}H]Chitin was kindly given by Dr. Judith Zyskind, San Diego State University, San Diego, CA, U.S.A. Swollen chitin from crab-shell granules was prepared by the method of Soto-Gil & Zyskind [14].

Methods

Growth and encystation of *E. invadens*. *E. invadens* strain IP-I [2] was obtained from Dr. Gordon Bailey (Morehouse University, Atlanta, GA, U.S.A.). The trophozoites were grown at room temperature for 7 days in Diamond TYI-S-33 medium [15] in plastic tissue-culture flasks. For encystation, trophozoites were harvested in the late-exponential phase of growth by first chilling the flasks in ice/water for 10–30 min, centrifuging at 1000 *g* for 10 min at ~ 10 °C, and then washing thoroughly with phosphate-buffered saline (PBS) to remove all nutrients. Trophozoites were then suspended at 5×10^4 cells·ml^{-1}, either in hypo-osmotic medium (distilled water/medium, 3:2, v/v) or in TYI-S-33 growth medium without added glucose [1]. Unless otherwise indicated, the cells were harvested after 2 days of incubation at room temperature, washed with PBS two or three times, and then stored as pellets at −70 °C until enzyme assay.

Cell fractionation. Cell pellets of ~ 3×10^8 cells were resuspended in 10 ml of assay buffer consisting of 50 mM-Hepes/NaOH, pH 7.0, 3 mM-$MnCl_2$, 1 mM-phenylmethanesulphonyl fluoride (PMSF) and 1 μM-*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) (a cysteine-proteinase inhibitor) and subjected to three or four freeze–thaw cycles to break the cells (checked microscopically) before homogenization in a Teflon homogenizer at 0–4 °C. The cells were then centrifuged at 500 *g* for 15 min to remove cell debris, unbroken cells and nuclei. The 500 *g* supernatant was then centrifuged at 15000 and 100000 *g*

Abbreviations used: ChS, chitin synthase (EC 2.4.1.16); GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; PBS, phosphate-buffered saline (KH_2PO_4, 1.5 mM; Na_2HPO_4, 8.1 mM; KCl, 27 mM; NaCl, 137 mM, pH 7.0); PMSF, phenylmethanesulphonyl fluoride; E-64, *trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane.

consecutively. The cell pellets were resuspended in the same buffer before use.

Assay of ChS activity. On the basis of preliminary studies, ChS activity was routinely determined with UDP-GlcNAc as substrate by a modification of the method of Kang *et al.* [5]. Unless otherwise specified, the assay mixture contained the following, in a final volume of 100 μl: 32 mM-GlcNAc, 1 mM-digitonin, 30 μl of enzyme (100–200 μg of protein) in the assay buffer described above. For pH profiles, buffers were: 50 mM-sodium acetate, pH 4.0–5.0; 50 mM-Tris/maleate, pH 5.0–7.0; 50 mM-Hepes/NaOH, pH 7.0–7.5; and 50 mM-Tris/HCl, pH 7.5–8.5. The reaction was initiated by addition of 1 mM-UDP-[^{3}H]GlcNAc (2×10^5 c.p.m. in 100 μl). After incubation for 1 h at 37 °C, the reaction was terminated by the addition of 0.8 ml of cold ethanol (80%, v/v). The pellet obtained by centrifugation (5000 *g*, 10 min) was suspended in 100 μl of distilled water and reprecipitated twice with the same volume of ethanol and dried under N_2. Finally, the precipitate was suspended in 100 μl of water, and incorporated radioactivity was counted in Aqua-Mix scintillation cocktail (New England Nuclear, Boston, MA, U.S.A.). Enzyme activity was expressed as nmol of [^{3}H]GlcNAc incorporated/h per mg of protein. Protein was measured by the procedure of Lowry *et al.* [16] with BSA as standard. Assays were performed in duplicate.

Purification of chitinase. Chitinase from *Serratia marcescens* was affinity purified by adsorption on chitin by the method of Roberts & Cabib [17]. The activity of purified chitinase was 15–30 munits/ml, and no proteinase activity was detectable as measured by digestion of azocasein or gelatin.

Isolation of ChS reaction product. Scaled-up enzyme reactions (total volume, 0.5 ml) were terminated by chilling the reaction tubes on ice and applying the contents to a Sephadex G-50 column (20 cm × 0.75 cm) which was previously equilibrated with 25 mM-Tris/HCl, pH 7.5. Two peaks were observed in the column profile, one in the void volume (V_0) and another near the retention volume (V_r) of the column. The fractions in the void volume, which were considered to be reaction product, were pooled and precipitated with 9 vol. of ice-cold acetone. Acetone-precipitable material was centrifuged at 5000 *g* for 15 min at 4 °C. The pellet was dried in a gentle stream of N_2 and resuspended in 0.5 ml of 50 mM-phosphate buffer, pH 6.5. An 80 μl portion of resuspended material was treated with 20 μl (0.6 munit) of purified chitinase for $\frac{1}{2}$ h or 2 h at 37 °C.

Chitinase digestion of ChS reaction product. Chitinase-digested material (100 μl) was centrifuged at 5000 *g* for 10 min and the supernatant (approx. 80–90 μl) was mixed with ~ 400–450 μl of 50 mM-phosphate buffer, pH 6.5, and applied to a Bio-Gel P2 column (0.75 cm × 100 cm) previously equilibrated with 0.1 M-acetic acid and eluted with 0.1 M-acetic acid. Fractions (1 ml each) were collected and the radioactivity was counted. To calibrate the column, standard chitotriose, chitobiose and GlcNAc were mixed with the chitinase-digested material and detected colorimetrically [18].

Digestion by cytohelicase. Aliquots of each fraction from the Bio-Gel-P2 column were assayed for radioactivity. Chitotriose and chitobiose peaks were located by colorimetric assay [18], pooled, mixed with 1 M-Na_2HPO_4 and treated with cytohelicase as a source of β-*N*-acetylglucosaminidase (100 μl of pooled fractions + 5 μl of cytohelicase stock solution, diluted 5-fold) for 12 h at 30 °C. The reaction was terminated by addition of 0.1 M-acetic acid, centrifuged at 5000 *g* for 10 min, and each supernatant was loaded on a Bio-Gel P2 column. Fractions were then counted for radioactivity. The GlcNAc standard was located by colorimetric assay [18].

Proteinase treatment. The soluble ChS reaction product, which was isolated by exclusion from Sephadex G-50, was treated with either 0.6 munit of chitinase or 20 munits of Pronase E (*Streptomyces griseus*) for 2 h at 37 °C (80 μl of reaction product in 50 mM-phosphate buffer, pH 6.5, and 20 μl of chitinase or Pronase E). The proteinase digestion was terminated by adding 5 μl of PMSF (final concn. 1 mM) to the reaction mixture. Chitinase- and proteinase-digested materials were centrifuged and the supernatants mixed with 400–450 μl of phosphate buffer before application to a Bio-Gel P2 column previously equilibrated with 0.1 M-acetic acid and eluted with 0.1 M-acetic acid. Fractions (1 ml each) were collected and assayed for radioactivity.

RESULTS

Encystation increases ChS activity

Since chitin is a major component of the *E. invadens* cyst wall, but is not present in the trophozoite, we wondered whether ChS activity increases during encystation. We induced encystation of *E. invadens* by two published procedures: glucose starvation [1] and dilution of the growth medium [2]. Fig. 1 shows that, with both procedures, the specific activity of ChS increased sharply (~ 8–10-fold) from 0 to 2 days of encystation and then decreased. In comparison, a lower and more gradual increase was observed in control cells cultured in complete growth medium.

Characterization of particulate and soluble ChS activity

Table 1 shows the relative distribution of ChS activity in crude membrane and soluble supernatant fractions. Unlike the ChS of yeast and other organisms [19], more than 60% of the enzyme activity in *E. invadens* was in the high-speed supernatant.

The rate of incorporation of label into ethanol-insoluble material was linear up to 1 h of incubation and then declined gradually (results not shown). The substrate–activity curve (Fig. 2*a*) shows that both soluble and particulate enzyme activities increased with increasing concentration of UDP-GlcNAc until concentrations of greater than 1 mM were reached, when the activities of both enzymes declined very sharply and were virtually absent at 2 mM. The double-reciprocal plots ($1/v$ versus $1/[S]$) of *E. invadens* ChS are sigmoidal, which may be due to a co-

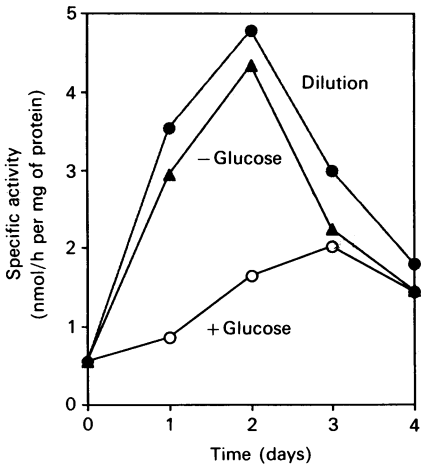

Fig. 1. Regulation of chitin synthase activity in encysting *E. invadens*

Parasite cultures (~ 5×10^4 cells/ml) were incubated for the times indicated in (i) glucose-deficient medium (▲), (ii) diluted growth medium (●) or (iii) normal growth medium (+ glucose, ○). Parasites were harvested and total ChS activity was measured in the 500 *g* supernatant as described in the Experimental section. The experiment was carried out in duplicate. Similar results were obtained in two other experiments.

Table 1. Relative distribution of ChS activity in particulate and supernatant fractions

Results presented here are the means for three different experiments, which differed by < 5%

Fraction	Total units (nmol/h)	Specific activity (nmol/h per mg of protein)	Recovery (%)
Crude (500 *g* supernatant)	544.6	12.8	100
15000 *g* pellet	158.0	14.5	29.0
100000 *g* pellet	58.3	9.1	10.6
100000 *g* supernatant	332.0	13.0	61.0

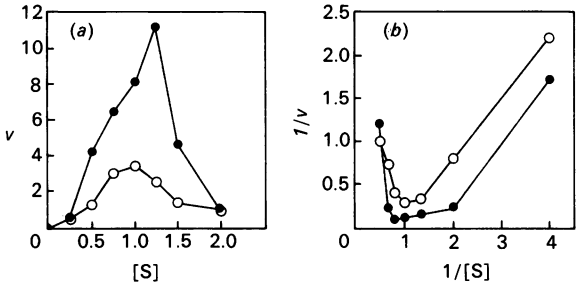

Fig. 2. Effects of substrate concentrations on particulate and soluble ChS activities

Particulate (○) and soluble (●) enzyme fractions were incubated with the indicated concentrations of UDP-GlcNAc (0.2–2.0 mM) as described under 'Methods'. (*a*) *v*-versus-[S] plot; (*b*) 1/*v*-versus-1/[S] plot. The K_m and $V_{max.}$ values were calculated from a linear 1/*v*-versus-1/[S]^2 plot (results not shown).

Table 2. Effect of bivalent-metal ions

The activity under standard conditions was taken as 100%: 3.8 nmol/h per mg of protein for the soluble enzyme and 5.8 nmol/h per mg of protein for the particulate enzyme. Results shown here are the means for two experiments which differ by ⩽ 8%.

Additions (at 2 mM)	Relative ChS activity			
	Soluble ChS		Particulate ChS	
	+3 mM-Mn^{2+}	−Mn^{2+}	+3 mM-Mn^{2+}	−Mn^{2+}
None	100	12	100	22
Mg^{2+}	53	15	37	26
Co^{2+}	235	56	221	2
Cu^{2+}	77	16	41	0
Fe^{2+}	65	9	43	0
Ca^{2+}	115	8	41	64

operative effect (Fig. 2*b*). However, apparent K_m and $V_{max.}$ values were determined from linear plots of 1/*v* against 1/[S]^2 [20]. The substrate affinity of the soluble ChS was slightly lower (apparent K_m 0.35 mM; $V_{max.}$ 4.16) than that of the particulate enzyme (K_m 0.23 mM; $V_{max.}$ 2.17) (results not shown). This difference is probably not significant. We do not know the concentration of UDP-GlcNAc in the cell or whether this changes during encystation.

We found that both the soluble and particulate ChS activities

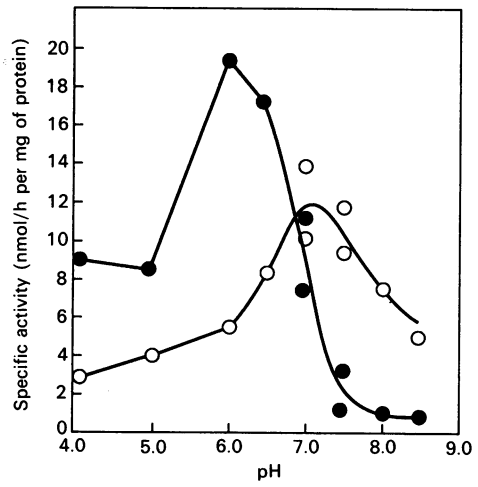

Fig. 3. pH–activity profiles of the ChS of *E. invadens*

Soluble (●) and particulate (○) cell fractions were prepared as described in the Experimental section. The enzyme activities were measured over the pH range 4.0–5.0 with 50 mM-sodium acetate, 5.0–7.0 with 50 mM-Tris/maleate, 7.0–7.5 with 50 mM-Hepes/NaOH, and 7.5–8.5 with 50 mM-Tris/HCl. Similar results were obtained in two separate experiments.

of *E. invadens* were most stimulated by Mn^{2+} (Table 2). Since Mn^{2+} and Co^{2+} may be as effective as Mg^{2+} in some systems [21], we tested these and other bivalent-metal ions in the presence or absence of Mn^{2+}. Co^{2+} stimulated the activity 2-fold in the presence of Mn^{2+} and could partly replace Mn^{2+} in the case of soluble ChS. However, other metal ions tested were either not effective or inhibitory. We do not yet know how Mn^{2+} affects the kinetic parameters of ChS.

One of the greatest differences we have observed between the soluble and particulate ChS of *E. invadens* is in their pH–activity profiles (Fig. 3). Whereas the particulate enzyme has a pH optimum of 7.0–7.5, the soluble enzyme is more active in a slightly acidic environment, namely pH 6.0.

Table 3 shows that UDP, a reaction product, as well as the substrate analogues UDP-glucose and UDP-GalNAc, were strongly inhibitory to both activities. In contrast, Polyoxin-D and Nikkomycin, which are potent inhibitors of yeast and fungal ChS [22,23] and block the encystation of *E. invadens* [24], did not effect *E. invadens* ChS activity (Table 3).

Preformed chitin inhibits ChS activity

Since nascent chitin is more susceptible to chitinase than cross-linked insoluble chitin polymer, we wondered whether the ChS product was being degraded by an endogenous chitinase activity, as has been shown for *Artemia* [13]. The addition of preformed chitin, to adsorb chitinase [17], to the assay medium inhibited the ChS activity of the membrane and soluble supernatant fractions (Table 3), suggesting that nascent chitin of *E. invadens* is not degraded by endogenous chitinase activity in our preparations.

Stimulation by digitonin and phospholipids

ChS I of *S. cerevisiae* is a particulate enzyme that requires the sterol-binding detergent digitonin both for initial solubilization and for the activity of the purified enzyme [5]. Therefore we assessed the effects of this detergent on the *Entamoeba* ChS activities. At concentrations up to 1 mg·ml^{-1}, digitonin stimulated the ChS activity approx. 2-fold (Table 4), but the activity was inhibited at higher concentrations (results not shown).

Since Braun & Calderone [11] demonstrated that phosphatidyl-

Table 3. Inhibition of ChS by UDP compounds

The activity under standard conditions was taken as 100% (5.4 nmol/h per mg of protein for soluble and 9.4 nmol/h per mg of protein for particulate enzymes). The data presented here are means of four different experiments, and variations were within 5%.

Incubation	Relative enzyme activity	
	Soluble	Particulate
Complete	100	100
plus 0.5 mM-UDP	0	0
plus 0.5 mM-UDP-glucose	14	37
plus 0.5 mM-UDP-GalNAc	5	0
plus Polyoxin-D (100 μg/ml)	100	95
plus Nikkomycin (100 μg/ml)	100	100
plus chitinase (0.6 munit)	0	0
plus chitin (0.2%)	50	72

Table 4. Effect of phospholipids

Particulate and soluble ChS were prepared as described in the Experimental section. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine. The activity under standard conditions was taken as 100% (8.0 nmol/h per mg of protein for soluble and 24.5 nmol/h per mg of protein for the particulate enzymes). The results are averages for two different experiments ±9%.

Additions (100 μg/ml)	Relative ChS activity			
	Soluble ChS		Particulate ChS	
	+Digitonin*	−Digitonin	+Digitonin*	−Digitonin
None	100	60	100	37
PC	85	84	37	132
PE	200	237	135	94
PI	66	79	151	166
PS	163	280	150	110

* 1 mM.

serine stimulated *C. albicans* ChS activity and proposed that, for maximal activity, the enzyme required lipid association, we tested the effects of various phospholipids on soluble and particulate ChS from *E. invadens*. Table 4 shows that phosphatidylethanolamine and phosphatidylserine (100 μg/ml) each stimulated the soluble enzyme activity significantly, in both the presence and absence of 1 mM-digitonin. Fig. 4 shows that phosphatidylserine also stimulated the particulate ChS activity at lower concentrations. Maximum stimulation (approx. 2-fold) was observed at 50 μg/ml and was not effective at higher concentrations (Fig. 4). By contrast, phosphatidylethanolamine stimulated the soluble ChS and had no influence on the particulate enzyme (Fig. 4), whereas phosphatidylinositol stimulated the particulate enzyme (Table 4).

Trypsin treatment

ChS I of *S. cerevisiae* is a zymogen which is activated by pretreatment with trypsin [5]. To test the possibility that the ChS of *E. invadens* is also a zymogen, we preincubated 90 μl of either particulate or soluble enzyme (prepared as described in the Experimental section, without proteinase inhibitors) with trypsin (40, 60, 80 and 100 $\mu g \cdot ml^{-1}$, final concns.) or buffer control for 15 min at 30°, before adding soybean trypsin inhibitor

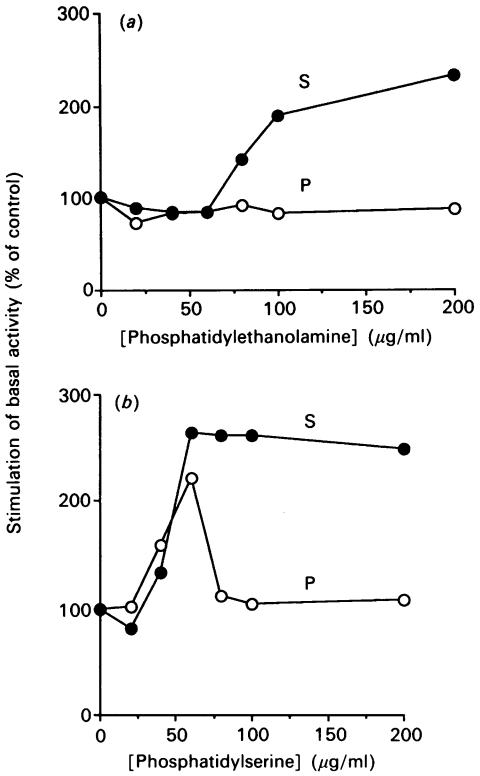

Fig. 4. Stimulation of ChS activity by phospholipids

ChS activities were measured in the presence of phosphatidylethanolamine (*a*) or phosphatidylserine (*b*) at the concentrations shown, using the standard assay conditions, except that digitonin was omitted. For the soluble (S) ChS (●), 50 mM-Tris/maleate buffer, pH 6.0, and for the particulate (P) (○) enzyme, 50 mM-Tris/maleate, pH 7.0 or 50 mM-Hepes/NaOH, pH 7.0, were used. Results are expressed as stimulation of activities where control activity (no phospholipid addition) was taken as 100%. Results presented are averages for two different experiments that differed by less than 9%.

(450 $\mu g \cdot ml^{-1}$), as described by Braun & Calderone [11]. Amoebal ChS activities were decreased, rather than increased, by trypsin treatment (results not shown).

Digestion of the reaction product by chitinase

Newly synthesized chitin is highly susceptible to chitinase cleavage before formation of interchain hydrogen bonds. Therefore we added affinity-purified *S. marcescens* chitinase to ChS assay mixtures. Fig. 5 shows that the presence of chitinase in the reaction mixture prevents the formation of ethanol-precipitable product in a concentration-dependent manner.

Characterization of the reaction product

The *E. invadens* ChS reaction product was insoluble in 80% (v/v) ethanol. For further characterization, the ethanol-insoluble ChS product was isolated by exclusion from a Sephadex G-50 column (see under 'Methods') and treated with purified chitinase for ½ to 2 h at 37 °C, and the chitinase-digested product was analysed on a Bio-Gel-P2 column. As Fig. 6 shows, chitinase generates radiolabelled di- and mono-saccharides, depending upon the duration of incubation. As described previously [25], prolonged digestion with chitinase yields some GlcNAc, attributable to the β-*N*-acetylglucosaminidase activity of purified chitinase. To confirm the identity of this product, we pooled the chitobiose peak from a Bio-Gel P2 column, treated it with cytohelicase, and re-chromatographed it on the same column.

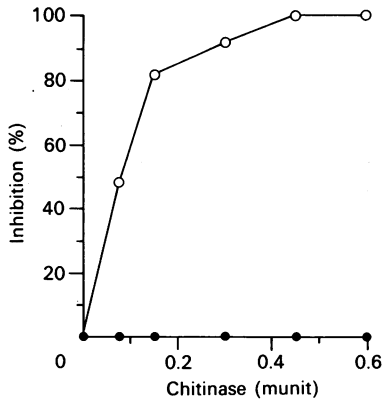

Fig. 5. Inhibition of product formation by chitinase

Incorporation of UDP-[^{3}H]GlcNAc into ethanol-insoluble product was measured in the presence of various amounts of affinity purified *S. marcescens* chitinase added to the reaction mixture (○). Boiled chitinase was used as a control (●). Similar results were obtained in two other experiments.

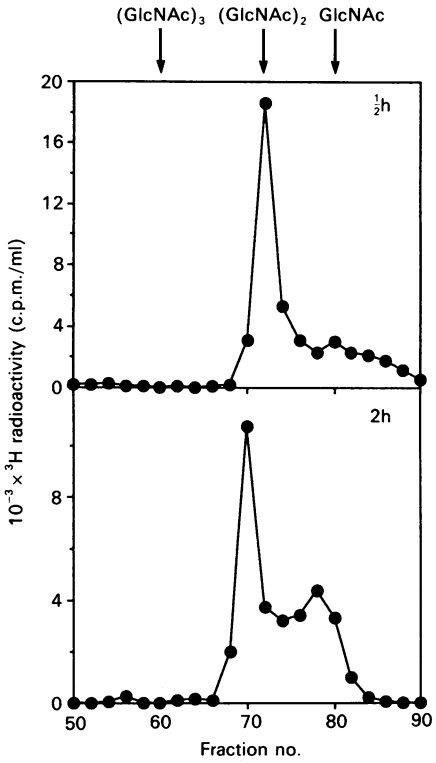

Fig. 6. Analysis of chitinase-digested *E. invadens* ChS reaction product on Bio-Gel P2

ChS (100000 *g* supernatant) reaction product was isolated by exclusion from a Sephadex G-50 column as described in the Experimental section. Acetone-precipitable material was treated with 0.6 munit of chitinase for ½ h and 2 h and the supernatants were separately chromatographed on Bio-Gel P2. Depending on the time of incubation, radioactive peaks migrated with the chitobiose and GlcNAc standards. Boiled chitinase was used in control experiments, and no radioactive peaks were detected. Similar results were found in three separate experiments.

Most of the chitobiose was degraded by the cytohelicase treatment and migrated with the GlcNAc marker on the Bio-Gel-P2 column (Fig. 7). When ^{3}H-labelled crab-shell chitin was digested

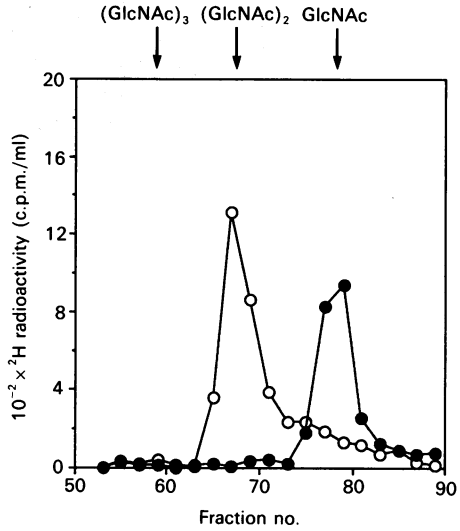

Fig. 7. Digestion by cytohelicase

E. invadens ChS reaction product was isolated by Sephadex G-50 column chromatography, digested with chitinase, and separated on a Bio-Gel P2 column as shown in Fig. 6. Fractions containing the chitobiose peak were pooled, neutralized with 1 M-Na_2HPO_4 and treated with cytohelicase for 12 h at 30 °C as specified in the Experimental section. The control materials treated with active or boiled cytohelicase were sequentially rechromatographed on the same Bio-Gel P2 column. After 12 h treatment with active (●), but not boiled cytohelicase (○), most of the radioactivity migrated with the GlcNAc standard. Similar results were obtained in two different experiments.

with chitinase followed by cytohelicase and chromatographed, similar products were obtained (results not shown). In each case, control incubations contained boiled enzymes. In contrast with digestion of the reaction product by chitinase, no chitobiose was released after incubation with a high concentration of proteinase (results not shown).

DISCUSSION

The present study demonstrates ChS activity in *E. invadens* and suggests that it may be a key enzyme in the synthesis of the cyst wall of encysting *E. invadens*. In addition to its importance in the differentiation of this parasite, ChS is a crucial enzyme in the life cycle of many higher invertebrates and fungi. Chitin, a polymer of $\beta(1 \rightarrow 4)$-linked GlcNAc, is a major component of certain protozoa, nematodes, arthropods and fungi [11,26]. For example, in *S. cerevisiae*, chitin forms the primary septum between dividing cells, and its biosynthesis is a temporal event under hormonal or α-factor pheromone control [26,27]. Chitin has a structural role in the primary egg-shell layer of many worms, and has been demonstrated in the sheath of gravid *Brugia malayi* females [28] and is also a major component of insect cuticle. A major insecticide, Dimilin (diflubenzuron), inhibits the ChS of the brine shrimp *Artemia salina* and morphogenesis of *B. malayi* [13,28]. Polyoxin-D, an analogue of UDP-GlcNAc, inhibits ChS and budding of *S. cerevisiae*.

Of the enzymes capable of catalysing the formation of an insoluble structural polysaccharide, ChS I of *S. cerevisiae* was the first to be extensively purified and characterized [5]. A second enzyme, ChS II, was also detected in *S. cerevisiae* [7,8] after the observation that normal amounts of chitin are made by strains which lack ChS I activity because of disruption of the ChS I gene. Silverman *et al.* [29] proposed that ChS II is essential for septum formation, whereas ChS I has a repair function. Recently Bulawa

& Osmond [9] reported that ChS I and ChS II are not required for chitin synthesis *in vivo* and proposed the existence of ChS III.

The present study demonstrates the presence of ChS in *E. invadens* which catalyses the incorporation of [^{3}H]GlcNAc from UDP-GlcNAc into a trichloroacetic acid- or ethanol-insoluble product. The identification of the product as chitin was shown by its digestion to chitobiose and GlcNAc by purified chitinase, but not by proteinase. Most interestingly, the ChS activity increased 8–10-fold in cells exposed to the negative stimuli of glucose deprivation and dilute medium, which induce encystation (Fig. 1). This is the first demonstration of a developmentally regulated enzyme in the genus Entamoeba.

The *E. invadens* ChS is unusual in many respects. In many organisms, ChS (e.g. ChS I and II of *S. cerevisiae*) exists in a zymogen form which is activated by proteolytic digestion. However, the ChS of *E. invadens* was not activated by trypsin pretreatment, like ChS III of *S. cerevisiae* [9] and ChS in the mycelial phase of *Blastomyces dermatitidis* [30]. The cell-fractionation experiment (Table 1) shows that approx. 60% of the enzyme activity is localized in a 100000*g* supernatant fraction. Therefore we tested the idea that particulate ChS may be released by endogenous proteinases during cell fractionation. However, the addition of proteinase inhibitors to the cell-fractionation and enzyme-assay buffer failed to influence the distribution of ChS, suggesting that the localization of a major proportion of the ChS activity in the high-speed supernatant is not due to proteolysis. It is possible, however, that an endogenous proteinase may have acted before it was inactivated by exogenous inhibitors.

Digitonin, a sterol-binding detergent which stimulates both particulate and purified forms of *S. cerevisiae* ChS I, also stimulates the *E. invadens* enzyme (Table 4). At present we do not understand why soluble ChS in *E. invadens* is stimulated by digitonin. Several studies of *N. crassa* and *Mucor rouxii* [31,32] indicated preferential localization of ChS in microvesicles called 'chitosomes'. It is possible that, in *E. invadens*, ChS is localized in similar structures. During cell disruption, ChS could be released from these vesicles as soluble enzyme. Further purification of soluble ChS will be necessary to define the roles of phospholipids and digitonin in stimulating soluble ChS activity in *E. invadens*. One possibility is that these amphipathic molecules may mimic the membrane milieu of the particulate enzyme. Alternatively, these detergents may affect enzyme conformation.

Polyoxin-D and Nikkomycin, which inhibit *S. cerevisiae* ChS and encystation of *E. invadens* [24], would be expected to inhibit ChS from *E. invadens*. However, our results show (Table 3) that neither the particulate nor the soluble ChS of *E. invadens* is inhibited by these peptide antibiotics. Previously, Horst [13] has shown that ChS from *Artemia salina* is also resistant to Polyoxin-D. We have found that our Polyoxin-D and Nikkomycin are inhibitory to *S. cerevisiae* ChS, even after preincubation with *E. invadens* extract. Thus it does not appear that these analogues are degraded by amoebal peptidases [33]. At present we do not know why *E. invadens* ChS is not inhibited.

The enzyme responsible for transferring GlcNAc from UDP-GlcNAc to form insoluble chitin would be essential for cyst wall formation and, therefore, transmission of amoebiasis. Although ChS was previously detected in encysting *E. invadens* [24], to our knowledge this is the first characterization of this enzyme in *Entamoeba* or in other parasitic protozoa. The high-molecular-mass reaction product was digested to chitobiose by affinity-purified *S. marcescens* chitinase, but not by proteinase, properties which are typical of chitin. Though very similar, two forms of ChS have been found in *E. invadens*. The major difference observed between the particulate and soluble forms is in their pH–activity profiles (optimal pH 6.0 as against 7.0–7.5) and stimulation by phospholipids. Recently Young & Robins [34] reported the presence of two distinct ChS activities with different pH optima in yeast and hyphal forms of *Candida albicans*. Further studies are now required to elucidate the relationship between these two forms of the enzyme. In the future, the purification and molecular cloning of this enzyme will help us to understand the mechanism of encystation and the role of ChS in cyst wall formation by *E. invadens*.

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